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TWO-PHASE KINETICS OF PROTON RELEASE FROM CHLOROPLASTS BY ACID–BASE TRANSITION

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SUMMARY

The kinetics of proton release in chloroplast suspensions by acid–base transition were studied by means of a stopped-flow spectrophotometer. The time courses of pH change show two-phase kinetics, fast and slow. The fast phase suggests the rapid release of protons from the thylakoid membranes, and the slow phase the efflux through the membranes of organic acid (and/or protons) previously absorbed from the initial acid medium.

INTRODUCTION

Subjecting broken chloroplast fragments first to acid, then to base, is known to cause a transient high-energy state which results in ATP formation[1]. It was suggested that energy in this experiment was supplied by the electrochemical activity gradient of protons across the thylakoid membranes[2]. An organic acid having entered the grana vesicles in the initial acid stage of the experiment serves as a proton reservoir in the base stage[3,4].

In a previous paper[5], the kinetics of efflux of acidity from chloroplasts (protons, succinic acid, or both) by the acid-base transition were studied by means of a stopped-flow apparatus containing a glass pH electrode. Immediately after mixing the pH values observed were as much as 0.6 unit more alkaline than the final equilibrium pH, then dropped to the equilibrium value over a period of several seconds. Time courses to the equilibrium pH showed apparent first-order kinetics with two components (the first and the second phases). However, the pH tracing at low temperature ($< 10°C$) showed a transient alkaline trend during the first fraction of a second before the first phase (Figs 2, 3 in ref. 5). Phenomena of this sort seemed to be due to a slow glass electrode system failing to respond to faster changes in actual pH.

In this report more rapid and precise time courses in pH changes were followed by using a stopped-flow spectrophotometer with bromocresol purple, which has been shown not to be bound to membranes, as a pH indicator[6,7] of the chloroplast suspensions.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

METHODS

Chloroplasts were isolated from market spinach as described previously[8], and finally resuspended at about 0.4 mg of chlorophyll per ml in 10 mM NaCl. 0.2 ml of this suspension was mixed with 0.2 ml of a solution at pH 4.0 containing 25 mM succinic acid and 75 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in a reservoir of the stopped-flow apparatus maintained at a constant temperature of 3.5°C. The acidified chloroplasts were incubated for 15–20 s, 0.25 ml of which were then rapidly picked up into one of the two syringes in the apparatus. The other syringe contained an equal volume of 30 μM bromocresol purple and NaOH solution sufficient to neutralize the succinic acid to bring the final pH to about 8.1–8.4. Variations of the reaction mixture components are indicated in the figures.

The stopped-flow spectrophotometer SPS-1 (Yanagimoto Mgf. Co.) was used for rapid mixing and the changes in the absorbance of bromocresol purple at 594 nm after the mixing were recorded using the memoriscope MS-5019 A (Iwasaki Tsushinki Co.). The two syringes were driven by compressed N_2 at 1–2 kg/cm^2 for mixing, and the dead time, *i.e.* the time from the beginning of mixing to first entering the light path of the measuring cell for spectrophotometry, was under 2 ms, usually 1–1.2 ms. The temperature was controlled at 3.5°C, unless otherwise mentioned.

The pH changes of the chloroplast suspension were calculated from the absorbance–pH curves of bromocresol purple at 594 nm; absorbance changes of more than 0.02 could be detected per 0.1 pH unit at the pH used. Further, the changes in H^+ concentration (ΔH^+, μequiv/ml) were calculated from the pH values according to direct titrations of the buffer capacities of the chloroplast suspensions with acid and with NaOH at each time of the experiments.

RESULTS AND DISCUSSION

Fig. 1 shows the results of an experiment in which one syringe contained 12.5 mM succinate at pH 4.0 with chloroplasts (0.2 mg chlorophyll/ml), and the other contained bromocresol purple and NaOH so that the final pH after mixing the contents of the two syringes was 8.4. Contrary to the previous results using a glass electrode[5], the tracing shows a sharp increase in H^+ concentration in the suspension immediately after mixing, followed by a slow increase as a function of time until an equilibrium was attained in about 10 s.

In Fig. 2, faster recordings of the same experiment as in Fig. 1, either with or without chloroplasts, are shown in order to observe the initial stage of the ΔH^+ after mixing. When no chloroplasts were present, there was no change in H^+ concentration, indicating efficient mixing within the time resolution of the apparatus (Curve C in Fig. 2). When chloroplasts were present, however, the tracing shows a clear two-phase time course consisting of a fast and a slow phase (Curve B in Fig. 2). One interpretation of the slow phase (the first phase in ref. 5, and see below) already presented is that the succinic acid (and/or protons), having entered the grana vesicles through the thylakoid at the initial acid stage, leaked out of the thylakoid by diffusion after mixing with base[5]. This interpretation is also supported clearly in the present experiment by the fact that twice the amount of H^+ was released with twice the concentration of chloroplasts (Curves A and A $\times$ 1/2 in Fig. 2). And the finding

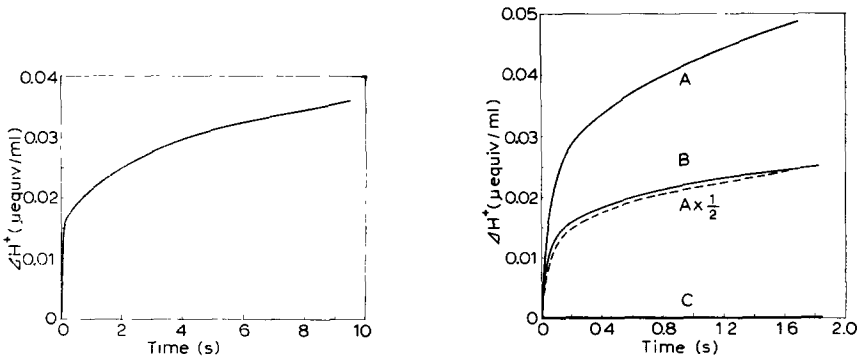

Fig. 1. Release of protons after mixing. The H^+ curve is reproduced from the oscillograph tracing of pH change (see text). Initial chloroplasts at pH 4.0 with 12.5 mM succinic acid and 38 μM DCMU The base solution contained 30 μM bromocresol purple and sufficient NaOH to neutralize the succinic acid to bring the final pH to 8.4. Chlorophyll concentration was 0.1 mg/ml after mixing.

Fig. 2. Fast recording of the release of protons as in Fig. 1 at two different chlorophyll concentrations (A, 0.2 mg chlorophyll/ml; B, 0.1 mg chlorophyll/ml) and without (C) chloroplasts. Chlorophyll concentrations indicated were those after mixing.

that the fast phase also increased 2-fold in Fig. 2 indicates that the fast phase of H^+ leakage with time is entirely due to the thylakoid membranes.

The H^+ curves with a clear distinction between the fast phase and the slow phase were sometimes observed as shown in Fig. 3. Addition of 5 mM NH_4Cl, an uncoupler of phosphorylation, at the acid stage caused a marked acceleration of proton efflux during the slow kinetic phase. Scarcely any change of the time course of the fast phase could be observed. Thus, the kinetic curves seem to represent a proton-releasing system of chloroplasts with two compartments in series[9], separated by two different barriers.

From the above experiments the simplest interpretation for the two-phase

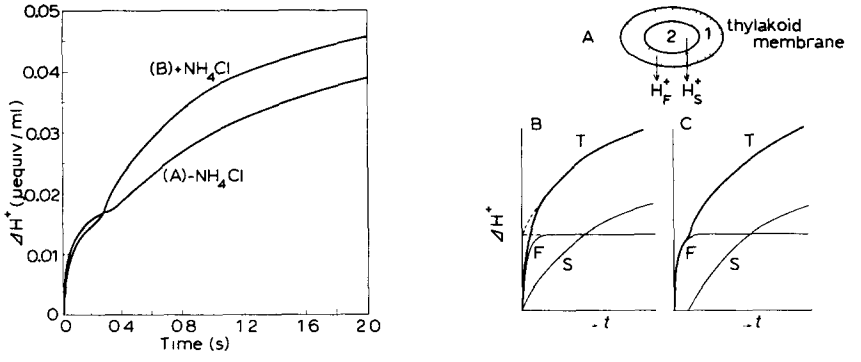

Fig. 3. Release of protons with and without NH_4Cl. Curve A, as for Fig. 2, but note the clear separation between the fast and the slow phases; Curve B, with 5 mM NH_4Cl at the acid stage. Chlorophyll concentration was 0.1 mg/ml after mixing. Initial pH 4.0, final pH 8.4.

Fig 4 . Diagramatic representation of proton release, plotted against time. (A) Possible sequence of proton release from thylakoid vesicle. (B) The fast and the slow phases start simultaneously after mixing. (C) The slow phase starts slightly later than the fast phase. 1, thylakoid membrane; 2, inside of thylakoid envelope; F, fast phase; S, slow phase; T, total time course

kinetics is that the H^+ curves consist of two kinds of independent proton-releasing processes as shown diagramatically in Fig. 4. When the release of protons in the slow phase starts slightly later than in the fast phase, a distinct separation of the two phases could occur as in Fig. 4C (*cf.* Fig. 3).

Both the fast and the slow phases followed apparent first-order kinetics when plotted on a semi-logarithmic scale. Rate constants thus obtained under different temperatures and with and without NH_4Cl are summarized in Table I. Practically no changes in the rate constants of the fast phase were observed, wheras those of the slow phase changed markedly under these conditions.

TABLE I

APPARENT FIRST-ORDER RATE CONSTANTS FOR THE TWO KINETIC PHASES

Experimental procedures are the same as in Fig. 1. Concentration of NH_4Cl was at the acid stage.

Expt	*Treatment*	*Rate constant* (s^{-1})	
		Fast phase	*Slow phase*
1	3.5 °C	35	0.43
	20 °C	37	1.07
2	None (3.5 °C)	18	0.56
	5 mM NH_4Cl (3.5 °C)	17	1.57

Insensitivity of the fast phase to temperature and NH_4Cl, together with its rapid rate (half-life of about 20–40 ms), suggests the dissociation of protons, which had previously entered and/or associated at the acid stage, from the thylakoid membrane itself (Compartment 1 in Fig. 4A), due to the sudden increase of environmental pH. This is further supported by the facts that (a) when the concentration of succinic acid at the acid stage was increased, the time course of the fast phase was not as strongly affected as that of the slow phase, and (b) the fast phase was also observed when the pH of the acid stage was adjusted to pH 4.0 with HCl, not with succinic acid.

Considering the size of the rate constants and their change by temperature and NH_4Cl in Table I, the slow phase in this experiment corresponds to the first phase in the previous paper[5]. The fact that the slow phase was followed by an additional, slower phase (corresponds to the second phase in ref. 5), at higher temperature and with NH_4Cl (not shown in Table I) also indicates the similarity. As described above, increased concentrations of succinic acid at the acid stage preferentially increased the ΔH^+ of the slow phase after mixing with base solution. Hence, it is concluded that the slow phase of the H^+ curve is due to the efflux of the succinic acid (and/or protons), which has entered the inside (Compartment 2 in Fig. 4A) of the thylakoid vesicles through the membranes by the acid–base transition. The relation of these two phases to the phosphorylation by acid–base transition remains to be established.

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REFERENCES

1 A. T. Jagendorf and E. Uribe, *Proc. Natl. Acad. Sci. U. S.*, 55 (1966) 170.
2 A. T. Jagendorf and E. Uribe, *Brookhaven Symp. Biol.*, 19 (1966) 215.
3 E. Uribe and A. T. Jagendorf, *Plant Physiol.*, 41 (1967) 697.
4 E. Uribe and A T. Jagendorf, *Arch. Biochem. Biophys.*, 128 (1968) 351.
5 Y. Nishizaki and A. T. Jagendorf, *Biochim. Biophys. Acta*, 226 (1971) 172.
6 J. B. Jackson and A. R. Crofts, *Eur. J. Biochem.*, 10 (1969) 226.
7 B Chance, A. R. Crofts, M. Nishimura and B. Price, *Eur J. Biochem.*, 13 (1970) 364.
8 Y. Nishizaki and A. T. Jagendorf, *Arch. Biochem. Biophys.*, 133 (1969) 255.
9 A. B. Hope, *Ion Transport and Membranes, A Biophysical Outline*, Butterworths, London, 1971, p. 16.